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Genetics of reproductive behaviour in Nasonia

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CHAPTER 5

Within-host-mating in the *Nasonia* genus is largely dependent on male behaviour

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ABSTRACT

Insects exhibit a wide variety of mating systems. For parasitoid wasps, mating is often confined to the environment where their hosts occur as these are used for oviposition and larval development. Many species mate at their emergence site where they emerge and in some cases mating occurs within the host prior to emergence. Parasitoids of the genus *Nasonia* parasitize blowfly pupae and typically mate immediately after emergence from the host puparium. We measured within-host-mating (WHM) frequencies in natural and laboratory populations of four *Nasonia* species that share the same fly species for parasitization. WHM is absent in *N. vitripennis*, rare in *N. longicornis* and *N. oneida*, but very frequent in *N. giraulti*. Interspecific crosses revealed that WHM rates in F1 hybrid females are intermediate to the pure species' values and that WHM frequencies were largely determined by the species of the males present in the host. Multiparasitization experiments were set up to test whether WHM has evolved in *N. giraulti* to prevent hybridization with *N. vitripennis*. Hosts that contained progeny of both *N. vitripennis* and *N. giraulti* resulted in lower WHM rates of *N. giraulti* when an exit hole was created by priorly emerged *N. vitripennis* males. This was also observed when an exit hole was artificially created in the host. We conclude that WHM is a male mediated trait in *N. giraulti*, resulting from males that refrain from making exit holes to mate with females inside the host. Frequently observed multiparasitization by *N. giraulti* and *N. vitripennis* in nature will reduce WHM in *N. giraulti*. This argues against the evolution of WHM as an effective prezygotic isolation mechanism between these two *Nasonia* species.

INTRODUCTION

A broad variety of mating systems occur in insects, which according to evolutionary theory have evolved to optimize the fitness of both sexes within a species. In general, one sex (usually the male) provides a signal to which the other sex (usually the female) responds (Andersson 1994). This often leads to competition between individuals of one sex and choosiness in individuals of the other sex. Both the preferences and preferred traits are the result of natural and sexual selection, and can be strengthened by adaptive evolution (Fisher 1930). In insects, mate discrimination often relies on the release of chemical and acoustic cues, such as sex pheromones and male calling, as part of the courtship display (Thornhill and Alcock 1983, Shuker and Simmons 2014). Sexual selection on mating traits is considered a driving force in speciation (e.g. Marie Curie SPECIATION Network 2012). There is now a large body of evidence that behaviour is a fast evolving trait in diverging populations, which can lead to prezygotic isolation (e.g. Coyne and Orr 2004). Closely related species, for example, often show divergent behaviour, particularly when they live in sympatry or have overlapping breeding periods, but do not hybridize in nature. Although the presence of such behavioural isolation is demonstrated quite easily, determining which behavioural traits are responsible for establishing this isolation proves to be more difficult (Bakker and Pomiankowski 1995).

Like all insects, parasitoid mating systems are influenced by the spatial and temporal distribution of females and males in nature as it predicts the availability of potential mates (Godfray 1994). Because parasitoids lay their eggs in other insects, in which their offspring develop, they are also dependent on the distribution and life cycle of their hosts. This has a large effect on the chance that individuals encounter mating partners of their own species, because the distribution of hosts often determines where mating occurs (Godfray 1994). This strong dependence to the biology of their hosts has led to a number of mating systems with aspects that are specific to parasitoids. Many parasitoid species mate at their emergence site, where males compete for available females, that disperse subsequently. Males and females may differ in dispersal behaviour because females need to search for new oviposition sites. Protandry, emergence of males before females, has evolved in many parasitoid species, because it gives the faster emerging males the opportunity to wait or actively search for the emerging females and mate with them upon emergence (van den Assem et al. 1980, Godfray 1994). Another behaviour found in some parasitoids is mating before emergence from the host, called within-host-mating (WHM). This behaviour has been observed in a few egg parasitoids (Dreyfus and Breuer 1944, Suzuki and Hiehata 1985), in the blowfly (*Protocalliphora*) pupal parasitoid *Nasonia giraulti* (Drapeau and Werren 1999), and some other species (see Godfray 1994).

The mating system of *Nasonia* has been studied extensively, including the effects of male size on reproductive success (Burton-Chellew et al. 2007b), occurrence of multiple mating (Burton-Chellew et al. 2007a, Leonard and Boake 2008), site fidelity and male aggression (Leonard and Boake 2006), female mate discrimination and within-host-mating (Drapeau and Werren 1999), the role of chemical communication (e.g. Steiner et al. 2006, Ruther et al. 2007), male mating behaviour (van den Assem and Werren 1994, Bordenstein et al. 2000, Beukeboom and van den Assem 2001), and local mate competition and sex allocation (Werren 1980, 1983, Grillenberger et al. 2009a, b, Steiner et al. 2009, Moynihan and Shuker 2011). Four species of *Nasonia* are known, of which three occur in sympatry in eastern North America, namely *N. vitripennis*, *N. giraulti* and *N. oneida*, where they occupy the same host patches and sometimes even share a host puparium during development (Grillenberger et al. 2009a). *N. longicornis* occurs in sympatry with *N. vitripennis* in western North America. *Nasonia* are protandric whereby males develop faster than females and, after eclosion, chew an exit hole in the host puparium to emerge from the host. Male *N. vitripennis* remain on the host and aggressively defend their position against other males at the exit holes until the females emerge. They often mate with the females emerging from the same host (Leonard and Boake 2006). Male *N. giraulti* do not show this fighting behaviour, but instead mate with the females within the fly host, before they emerge (Drapeau and Werren 1999). Leonard and Boake (2006) have shown a negative correlation between the level of aggression and WHM in the *Nasonia* genus. Drapeau and Werren (1999) reported that WHM is a frequent behaviour in *N. giraulti*, but is absent in *N. vitripennis* whereas *N. longicornis* shows intermediate levels. Drapeau and Werren (1999) proposed that WHM has evolved in *N. giraulti* and *N. longicornis* as a barrier to interspecific hybridization in sympatric populations with *N. vitripennis*. Additionally, they suggested that WHM might increase local mate competition - as mating occurs within the host, thereby increasing competition between brothers for mates – and lead to a more female-biased sex ratio. Leonard and Boake (2006) also found low levels of WHM in *N. vitripennis* and *N. longicornis*, and all *N. giraulti* females mated inside the host. They hypothesized that WHM is a female trait, because the number of males present within a host did not alter the frequency of WHM.

Here we present data on WHM rates of different field-collected populations of the four known *Nasonia* species, expanding on the earlier studies of Drapeau and Werren (1999) and Leonard and Boake (2006). Firstly, our study provides new data on WHM in the recently discovered *N. oneida* species (Raychoudhury et al. 2010a). Secondly, prompted by the fact that WHM has never been tested in the field, we investigate whether WHM is not solely induced by laboratory conditions, but a behaviour that also occurs under natural conditions. Thirdly, by setting up interspecific crosses between various species combinations under laboratory conditions, we investigate how WHM is inherited,. We address the hypothesis that WHM can act as a prezygotic isolation mechanism in *N. giraulti*,

by measuring WHM in multiparasitized hosts containing both *N. vitripennis* and *N. giraulti* individuals. Our results indicate that factors, other than preventing interspecific mating, must have played a role in the evolution of WHM in *Nasonia*. We consider whether WHM is a female or male mediated trait in *N. giraulti*, and discuss its possible adaptive significance in the context of species isolation.

Material & Methods

Lines and culturing conditions

Laboratory lines

The following *Nasonia* laboratory lines were used in one or more experiments, with origin between brackets. *N. vitripennis:* HvMIX3 (Hoge Veluwe, the Netherlands, 2001, described in van de Zande et al. 2014), Sal 8 (Ithaca NY, USA, 2007), ITH-4C (Ithaca NY, USA, 2006), ASymC (Leiden, the Netherlands, 1971), the red-eye mutant line STDR-TET; *N. giraulti*: NGVA-2 (Virginia NY, USA, 2006), NGPA-233F (Pennsylvania, USA, 1989), RV2 (Virginia, USA, 1986), and NGmix (consisting of five *N. giraulti* lines: RV2, NGVA-2, NGVA-7B (Virginia, USA, 2006) NGPA-233F, and NGNY6A5 (New York, USA, 2005)); *N. oneida* NONY 11/36-TET (Brewerton NY, USA, 2005); *N. longicornis*, RNLMN8510 (Minnesota, USA, 1989), IV7 (Utah, USA, 1986). After collection the lines have been maintained in the laboratory in diapause (approximately one generation per year) until the start of the experiments, except for NGmix, HvMIX3, ASymC, STDR-TET and RV2 which have been cultured in a 20ºC climate chamber since the lines were created.

Field lines

In addition to these laboratory lines, wasps of all four species were collected from the field in 2011 (Table S5.1), and immediately tested for within-host-mating in the laboratory. Iso-female lines were established either from single females collected directly from the field, or from a single female that emerged from a field-collected host. Single female wasps were isolated in a plastic vial and supplied with hosts. Resulting offspring formed the iso-female line which was maintained in mass culture vials at 20ºC and L16:D8.

For *N. vitripennis*, we have investigated 11 iso-female lines that were established from five nests in Davidson (North Carolina, USA), 30 iso-female lines that were established from 24 nests in Ithaca (New York, USA), 10 iso-female lines established from one nest in Lancaster (Pennsylvania, USA), and 7 iso-female lines from two nests in Kingston (Ontario, Canada). For *N. giraulti*, 63 iso-female lines have been investigated that were established from 35 nests in Davidson (North Carolina, USA), one iso-female line from one nest in Ithaca (New York, USA), and 12 iso-female established from three nests in Waynesboro (Virginia,

USA). For *N. longicornis*, four iso-female lines have been investigated that were established from one nest in Ithaca (New York, USA). A detailed description of the field lines, including the origin of the iso-female lines, can be found in Table 1.

During the experiments, all wasps were cultured in a 25°C climate chamber, with a 16:8 hrs light-dark cycle and 45% relative humidity. Under these conditions the generation time is approximately 14 days for *N. vitripennis*, and 16 days for *N. giraulti*, *N. longicornis* and *N. oneida*. Virgin females were collected 12-14 days after egg-laying. *Calliphora spp.* fly pupae were used as hosts for oviposition.

WITHIN-HOST-MATING (WHM) PHENOTYPES

Laboratory hosts

To determine WHM rates, mated females were provided with a single, fresh host every 24 hours for 5 days. Each parasitized host was incubated in an individual plastic test tube (60mm long, diameter 10mm) for thirteen days. At the expected day of emergence, hosts were inspected at regular intervals (approximately every 15 minutes) during the light phase. When the first emerged female was observed, the host was opened under CO_2 to anaesthetize the wasps, and ten females were randomly collected from within the host and placed individually in plastic tubes. The CO_2 anaesthetisation was used to prevent females from mating during the collection and was found not to influence the behaviour of the wasps (data not shown). The ten collected females were fed 10% concentrated sugar water for 24 hours, and subsequently provided with two hosts for parasitization. Their progeny was scored for the presence of females; if daughters were found, the test female was scored as having mated inside the host prior to emergence. If only males were produced, the test female was scored as virgin. The percentage of WHM was calculated by determining the number of test females (out of 10) that produced daughters. Our method of scoring WHM differs slightly from Drapeau and Werren (1999), Leonard and Boake (2006), and Ruther et al. (2014). Drapeau and Werren (1999) waited until 15-30% of the experimental hosts had emerged, and then started collecting the females from within the remaining intact hosts. Compared to our method, this could underestimate WHM rate, as their collection method could include females that were too young to have mated. Leonard and Boake (2006) and Ruther et al. (2014) collected each female immediately after emerging from the host. While this might give a more accurate result than our method, it is much more time consuming. In a pilot experiment with *N. giraulti* we did not find a difference in WHM rate between their and our method (results not shown). In *N. vitripennis*, we found that that our method shows lower numbers of WHM, as some females stay inside the host and emerge later.

Field-collected hosts

To test whether WHM also occurs under natural conditions, and is not a laboratory induced behaviour, 100 hosts parasitized by *N. giraulti*, *N. vitripennis* or *N. longicornis* were collected from 27 bird nests at field sites in Ithaca NY, USA, in the summer of 2011. Collected hosts were treated and scored similarly for WHM as the laboratory hosts, as described above. For this experiment, all females collected from within the hosts were hosted to determine the percentage of WHM.

INTERSPECIFIC CROSSES

To investigate the inheritance pattern of WHM, interspecific crosses were set up, using laboratory lines that had been cured from their cytoplasmic incompatibility-inducing *Wolbachia* bacteria (Breeuwer and Werren 1990): for *N. giraulti* the NGVA-2-TET line was used, for *N. longicornis* RLMN8510 and for *N. oneida* NONY 11/36-TET. A virgin female of one species was mated to a male of another species to generate hybrid females with a 50:50 genomic composition of the two species. Two days after mating, females were provided with hosts, which were scored for WHM as described above. Due to haplodiploidy, parasitized hosts contained F1 hybrid females that developed from fertilized eggs, and haploid non-hybrid males of maternal species origin from unfertilized eggs.

MULTIPARASITIZATION EXPERIMENT

In order to test whether *N. giraulti* females still mate within the host when another species is also present in that host, a multiparasitization experiment was set up. Hosts were simultaneously parasitized by a mated female from the *N. vitripennis* STDR-TET line and a mated female from the *N. giraulti* NGVA-2-TET line. This yielded hosts containing *N. vitripennis* females and males, together with *N. giraulti* females and males. Because the STDR-TET females have red eyes, instead of wildtype purple eyes, it was possible to distinguish the species among the female offspring. Test hosts were observed and treated as described above. When the first *N. giraulti* female emerged from the host, ten *N. giraulti* females were collected from the hosts to determine their WHM rate.

To test whether *N. giraulti* females would still mate within the host if there is an exit hole available resulting from a prior emergence of *N. vitripennis* males, we conducted a follow-up experiment in which an exit hole was artificially created with a needle 3 days prior to emergence.

STATISTICS

Within-host-mating data were analysed using generalized linear models with a binomial error structure. The species variable was fitted as fixed effect; geographic location and iso-

female line as random effect. For the results of the interspecific crosses, cross type was fitted as random effect. For the results of the multiparasitization experiment, treatment (multiparasitized vs non-multiparasitized; with or without hole created) was fitted as random effect. The significance of each factor was tested by comparing models, which either included or excluded species as an explanatory variable, using likelihood ratios tests (LRT) based on a $\chi 2$ distribution. Post-hoc tests were performed with a multiple comparison analysis and Tukey Contrasts. All statistical analyses were performed using R software (version 3.0.2; R Development Core Team, 2013).

RESULTS

WITHIN-HOST-MATING INCIDENCE

WHM rates were scored in eight different laboratory lines. There was a significant effect of species (χ^2, N=146, df=3, LRT=1035.2, p<<0.0001). Two of the three tested *N. vitripennis* laboratory lines (Sal 8 and ITH4C) show complete absence of WHM, whereas the third line HvMIX3) has a very low proportion of 0.6 ± 0.2% (Figure 5.1). The *N. oneida* line and the *N. longicornis* line show a low proportion of WHM, 9.9 ± 3.8% and 13.1 ± 3.4%, respectively. The *N. girault*i lines have a high proportion of WHM, with one line exhibiting 100% (NGPA233F), and the other lines 96.3 ± 2.9% (NGVA-1-TET) and 92.7 ± 2.9% (NGmix) of WHM.

Figure 5.2 summarizes the WHM proportions of field lines that were tested immediately after collection by allowing the females to parasitize laboratory hosts. The results are consistent with the laboratory lines; *N. vitripennis* has a low average proportion of WHM of 7.8 ± 17.4% (N=52 iso-female lines), *N. longicornis* shows an intermediate proportion of 40.7 ± 30.6% (N=4 iso-female lines), and *N. girault*i has a high WHM proportion of 91.7 ± 17.4%

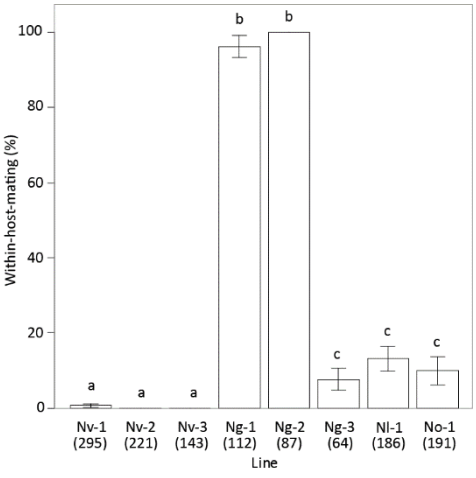

Figure 5.1 Within-host-mating rates in laboratory lines of four *Nasonia* species. Nv = *N. vitripennis*, Ng = *N. girault*i, Nl = *N. longicornis*, No = *N. oneida*. Nv-1 = HvMIX3, Nv-2 = Sal8, Nv-3 = ITH4C, Ng-1 = NGVA-1-TET, Ng-2 = NGPA233F, Ng-3 = NGmix, Nl-1 = RNLMN8510, No-1 = NONY 11/36. Sample size between parentheses indicate the number of individuals tested per line. Statistically significant differences (P<0.05, multiple comparison GLM, binomial) are indicated by non-capital letters. Error bars represent standard error.

(N=63 iso-female lines). There is a significant difference between the species (χ^2, N=397, df=2, LRT= 2994.9, p<<0.0001), and a significant interaction between species and geographical locality (χ^2, N=397, df=1, LRT=13.13, p= 0.0003). The latter points at the presence of genetic variation for WHM within field populations.

Figure 5.3 shows the WHM frequencies in field-collected hosts parasitized by either *N. vitripennis* or *N. girault*i. These are consistent with the data of the lab lines; *N. vitripennis* has lower proportions of WHM than *N. girault*i (4.0 ± 9.3%, N=90 and 79.3 ± 21.7%, N=50 respectively), and the difference between the two is significant (χ^2, N=95, df=1, LRT=56.12, p<<0.0001).

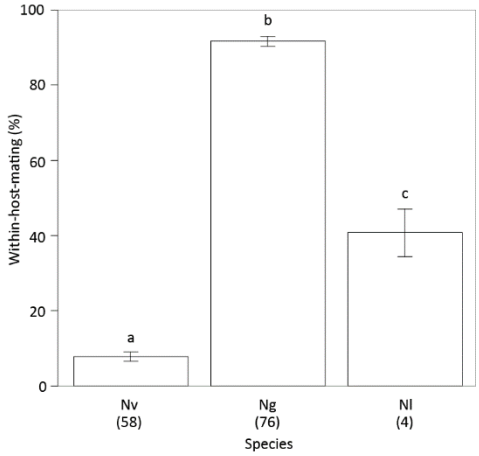

Figure 5.2 Within-host-mating rates in recently collected field lines of three *Nasonia* species. Nv = *N. vitripennis*, Ng = *N. girault*i, Nl = *N. longicornis*. Sample size between parentheses indicate the number of lines tested. Statistically significant differences (P<0.05, multiple comparison GLM, binomial) are indicated by non-capital letters. Error bars represent the standard error.

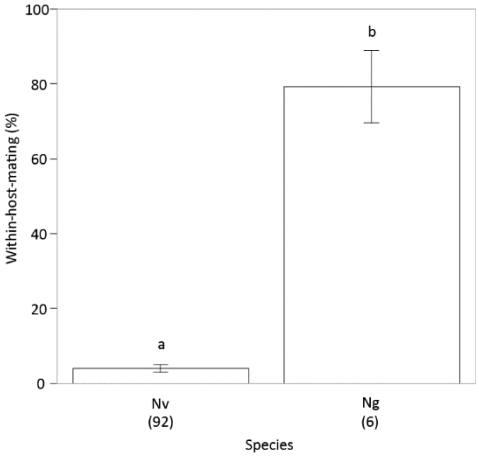

Figure 5.3 *Within-host-mating rates in naturally collected hosts from Ithaca, New York, USA. Nv = N. vitripennis, Ng = N. girault*i*. Sample size between parentheses indicate the number of hosts tested. Error bars represent the standard error.*

WITHIN-HOST-MATING IN F1 INTERSPECIFIC HYBRID FEMALES

To investigate the interspecific inheritance pattern of WHM, reciprocal crosses between species were set up and the WHM proportions were measured for F1 hybrid females from three different species pairs. *N. longicornis* / *N. girault*i (*NlNg* and *NgNl*) hybrid females had significantly different proportions of WHM, dependent on the direction of the cross (Tukey, N=28, df=1, z-value=6.40, p<0.0001; Figure 5.4A). Hosts containing Nl/Ng hybrid females with *N. girault*i males show a high WHM proportion (95.6 ± 5.3%, N=132), similar to hosts containing pure *N. girault*i (97.9 ± 4.5%, N=91) (Tukey, N=24, df=1, z-value=-0.91,

p=0.79). Hosts containing *NgNl* hybrid females with *N. longicornis* males show an intermediate proportion of WHM (53.1 ± 36.7%, N=122), which is significantly different from both pure *N. longicornis* crosses (2.6 ± 6.3%, N=101; Tukey, N=26, df=1, z-value=-5.47, p<0.0001) and pure *N. giraulti* crosses (Tukey, N=24, df=1, z-value=-4.97, p<0.0001). Additionally, WHM rates are significantly higher in hosts containing *N. giraulti* males (offspring from *N. giraulti* mother), compared to hosts containing *N. longicornis* males (offspring from *N. longicornis* mother; χ^2, N=50, df=1, LRT=245.12, p=<<0.0001). These results suggest that the presence of *N. giraulti* males mainly determines the proportion of WHM.

N. oneida / *N. giraulti* (*NoNg* and *NgNo*) hybrid females yielded high WHM proportions in both reciprocal crosses (Figure 5.4B): Hosts with hybrid *NgNo* females and *N. oneida* males show a WHM proportion of 69.2 ± 21.7% (N=39), and hosts with hybrid *NoNg* females and *N. giraulti* males show a proportion of 82.1 ± 17.6% (N=140). WHM frequencies differed between the F1 hybrid *N. oneida* / *N. giraulti* females of the reciprocal crosses: Females from a *N. giraulti* father and a *N. oneida* mother (*NgNo*) mated significantly less within the host compared to females from a *N. oneida* father and a *N. giraulti* mother (*NoNg*). F1 hybrid *N. oneida* / *N. giraulti* females showed an intermediate pattern of WHM compared to the paternal species which was significantly different (Tukey: *NoNg* hybrid compared to pure *N. giraulti*: N=38, df=1, z-value=-4.51, p<0.001; *NoNg* hybrid compared to pure *N. oneida*: N=56, df=1, z-value=-9.21, p<0.001; *NgNo* hybrid compared to pure *N. giraulti*: N=55, df=1, z-value=-6.26, p<0.001; *NgNo* hybrid compared to pure *N. oneida*: N=73, df=1, z-value=-8.57, p<0.001). In this species pair, WHM proportion is also significantly higher in hosts containing *N. giraulti* males compared to hosts containing *N. oneida* males (χ^2, N=111, df=1, LRT=245.12, p<<0.0001). The results in this species pair correspond to the observation in the *N. giraulti* / *N. longicornis* species pair, that high WHM proportions are the result of male *N. giraulti* individuals in the host.

N. oneida / *N. longicornis* (*NoNl* and *NlNo*) hybrid females yielded low WHM rates in both reciprocal crosses. There is a significant difference in the WHM proportion for the two reciprocal crosses (*NoNl* vs *NlNo*; Figure 5.4C; Tukey, N=26, df=1, z-value=3.25, p=0.005): Hosts containing hybrid *NoNl* females with *N. longicornis* males show a significantly higher WHM proportion (31.2 ± 28.4%, N=96) than hosts containing hybrid *NoNl* females with *N. oneida* males (4.7 ± 13.2%, N=46). The WHM proportion of hybrid *NoNl* females with *N. longicornis* males is significantly higher than in pure *N. longicornis* crosses (14.3 ± 12.8% (N=142); Tukey, N=33, df=1, z-value=3.75, p=0.001), but not different from pure *N. oneida* crosses (48.2 ± 22.3% (N=46); Tukey, N=34, df=1, z-value=1.96, p=0.187). The WHM proportion of hybrid *NlNo* females that were collected from hosts containing *N. oneida* males is significantly lower than from pure *N. oneida* crosses (Tukey, N=24, df=1, z-value=4.18, p<0.001) but not different from pure *N. longicornis* crosses (Tukey, N=23, df=1, z-value=-1.33, p=0.526). Additionally, the WHM proportion is significantly different between hosts that

contain *N. oneida* males and hosts that contain *N. longicornis* males (χ^2, N=57, df=1, LRT=13.20, p=0.0003). The results in this species pair suggest that the presence of *N. longicornis* males in the host increase WHM proportions. Additionally, differences between observed frequency of WHM in the reciprocal crosses, for all these species pairs, could be the result of differences in female mate discrimination in the F1 hybrid females (see discussion).

In summary, these results reveal that the WHM proportions in F1 hybrid females from interspecific crosses are intermediate to the proportion of WHM of the pure species crosses. For each investigated species pair, a significant difference was found between the reciprocal crosses, which is most likely caused by the male species present in the host. When *N. giraulti* males are present in the host, there always is a strong increase in WHM rate, indicating that WHM is a behaviour mainly mediated by *N. giraulti* males.

MULTIPARASITIZATION

To test whether *N. giraulti* females also mate inside the host when that host has been co-parasitized by another species, WHM was measured in hosts containing males and females of both *N. giraulti* and *N. vitripennis*. Females collected from these multiparasitized hosts showed a WHM proportion of 16.1 ± 33.7% (N=102), which is significantly lower than the WHM proportion of females from hosts parasitized by *N. giraulti* only (96.3 ± 2.9%, N=112; Tukey, N=34, df=1, z-value=-7.84, p<0.0001). Notably, the *N. giraulti* females and males emerged from the host simultaneously with the *N. vitripennis* females, which is almost two days earlier than normal for *N. giraulti*. These results clearly indicate that *N. giraulti* individuals used the exit hole created by the faster developing *N. vitripennis* males. To further examine this, an experiment was set up in which artificial exit holes were created in the hosts. In both *N. giraulti* lines tested, WHM proportions of females from such manipulated hosts were significantly lower than those of females from control hosts (0.6 ± 2.9%, N=102 for manipulated vs 96.3% for control hosts; Tukey, N=39, df=1, z-value=7.39, p<0.0001). These results confirm that WHM proportions depend on the presence of an exit hole, and show that *N. giraulti* males, by refraining from making exit holes in the host, they mate with females inside the host.

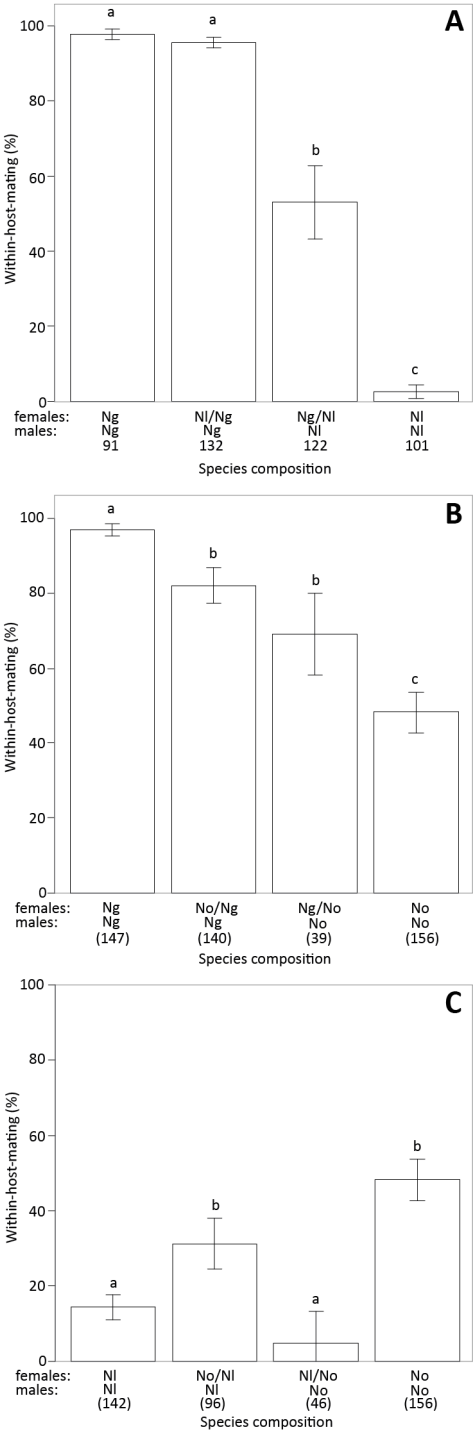

Figure 4 Within-host-mating rates in interspecific hybrids. (A) *N. longicornis* and *N. giraulti*. B) *N. oneida* and *N. giraulti*. C) *N. oneida* and *N. longicornis*. Ng = *N. giraulti*,; Nl = *N. longicornis*, No = *N. oneida*. The composition of the host includes the F1 hybrid females and pure species males. Sample size between brackets indicates number of individuals tested. Statistically significant differences (P<0.05, multiple comparison GLM, binomial) are indicated by non-capital letters. Error bars represent the standard error.

Discussion

We investigated the occurrence and inheritance of within-host-mating (WHM) in the *Nasonia* genus. We found absence or very low proportions of WHM in *N. vitripennis*, high WHM frequencies in *N. giraulti* and low frequencies in *N. longicornis* and *N. oneida* laboratory lines. This study is the first to report WHM proportions in *N. oneida*. Our data show that *N. giraulti* is the only species with high WHM rates and despite its phylogenetically close distance (Raychoudhury et al. 2010a), WHM is rare in *N.oneida*. This suggests that WHM has evolved recently in *N. giraulti*, after the split from *N. oneida*, or was secondarily list in *N. oneida*.

The results from *N. vitripennis*, *N. longicornis* and *N. giraulti* confirm previous results by Drapeau and Werren (1999) and Leonard and Boake (2006). However, some discrepancies between the studies have been found, as the *N. giraulti* females in our study tend to have a higher WHM frequencies overall, especially compared to the results of Drapeau and Werren (1999). This is most probably the result of different scoring methods. Our method based on anaesthetization of females, ensured that all females were tested at a specific moment, whereas the method used by Drapeau and Werren could include younger females which have a higher chance of being virgin due to the fact that they had less time to mate.

Newly collected wasp lines, as well as hosts collected directly from the field yielded similar WHM rates for *N. vitripennis* and *N. giraulti*. This means that WHM is not a laboratory induced behaviour and also occurs frequently in nature for *N. giraulti*, but not for *N. vitripennis*.

Interspecific crosses between species that differ in WHM rates were set up to determine how WHM inherits, in terms of dominance and sex-specificity. To reduce effects of interspecific mate discrimination, only species combinations were used that show little interspecific mate discrimination (Giesbers et al. 2013). This excluded testing the combination of *N. giraulti* and *N. vitripennis* that differ strongest in WHM proportion, as *N. vitripennis* females (and subsequent hybrids) show strong discrimination against *N. giraulti* males. Crosses between *N. giraulti* and *N. longicornis*, as well as *N. giraulti* and *N. oneida*, yielded F1 hybrid females with intermediate frequencies of WHM. Although this may suggest additive genetic variation for WHM, caution needs to be exerted for this conclusion because of the complex nature of the species interactions. Direct comparison of the two reciprocal hybrid crosses is hampered by changes in three variables at the same time, i.e. the male species present in the host, hybrid female choice towards that male species, and dysgenic effects in the hybrid females, and the added possibility of interactions between these three variables. Because of haplodiploidy, crosses between a *N. longicornis* female and a *N. giraulti* male yield progenies consisting of hybrid females with a 50:50 composition of *N. longicornis* and *N. giraulti* genome, and pure *N. longicornis* males. Reciprocal crosses,

between a *N. giraulti* female and a *N. longicornis* male, yield progenies with similar hybrid females, but pure *N. giraulti* males. The latter cross resulted in higher WHM rates which suggests an effect of the male species present in the host, consistent with the observation that *N. giraulti* shows higher WHM than *N. longicornis*. However, a possible alternative explanation is that hybrid females discriminate stronger towards *N. longicornis* males than towards *N. giraulti* males.

Although we have no data on mate discrimination in *N. giraulti* / *N. longicornis* hybrids against either *N. longicornis* or *N. giraulti* males to verify this, mate discrimination between pure *N. giraulti* and *N. longicornis* is low (Giesbers et al 2013; Buellesbach et al 2014). In the hybrid cross between *N. oneida* and *N. giraulti* we found similar significantly elevated WHM rates when *N. giraulti* males were present in the host. Another possible complication is that even though the hybrid female offspring of two reciprocal crosses are genetically identical, they differ in cytoplasm that is of maternal origin. Mitochondria and cytonuclear interactions may affect mate discrimination behaviour of hybrid females. Again we think that these effects are not so strong that they can explain the large difference in WHM rates, as mate discrimination rates between the pure species are low and rather similar for both reciprocal crosses (Giesbers et al 2013). Our results of the interspecific crosses therefore indicate that WHM is mediated by two effects, a genetic component that is dependent on species as the female hybrids show intermediate patterns of WHM and an effect of male species present in the host.

An effect of the male species present in the host was also found in multiparasitized hosts: significantly fewer *N. giraulti* females mated within the host, when both *N. vitripennis* and *N. giraulti* males were present. A likely explanation for this result is that faster developing *N. vitripennis* males chew an exit hole in the puparium wall soon after eclosion, through which the *N. giraulti* males and females also emerge directly. This shortens the time period that the *N. giraulti* males can mate with *N. giraulti* females inside the host. To test the validity of this explanation, artificial exit holes were created in hosts that were parasitized by *N. giraulti* only. This also led to a significant decrease of WHM, indicating that the timing of the creation of exit holes is an important factor in determining WHM behaviour. Apparently, *N. giraulti* males are able to force *N. giraulti* females to mate inside the host by waiting to create an exit hole. Interestingly, we also observed synchronization in emergence time in the multiparasitization experiment, suggesting that *N. giraulti* individuals speeded up their development in response to the presence of *N. vitripennis*.

Protandry, the faster development of males than females, is a widespread phenomenon in insect mating systems (Thornhill and Alcock 1983). The *Nasonia* system is a clear illustration of this behaviour: males have a shorter developmental time than females (1 to 2 days at 25°C) (Whiting 1967), and this allows them to leave the hosts first and compete at the exit hole for access to the later emerging females. Although both species are

protandric, the *N. giraulti* mating system is slightly different from that of *N. vitripennis*, as *N. giraulti* males do not emerge upon eclosion but remain in the host to mate with the females, only chewing the exit holes after mating. This indicates that WHM is a behaviour mediated by the males, and not the females, as has been suggested by Leonard and Boake 2006. Moreover, WHM appears to be correlated with site fidelity as *N. longicornis* and *N. oneida* both have intermediate rates of WHM and males do not tend to stay at the exit hole to wait for emerging females.

Drapeau and Werren (1999) proposed that WHM evolved in *N. giraulti* as a means of preventing hybridization with *N. vitripennis* in areas of sympatry. The results from our multiparasitization experiment, however, indicate that WHM is not an efficient prezygotic isolation mechanism between these two species. Hosts parasitized by both species result in a lower WHM for *N. giraulti* females than hosts that are exclusively parasitized by *N. giraulti*. Although limited information is available about the frequencies of multiparasitization in nature, Grillenberger et al. (2009a) showed that in the Ithaca (NY) area, *N. giraulti* was only found in bird nests that were also parasitized by *N. vitripennis*. Perez-Villa et al. (in prep.) found that *N. giraulti* females prefer to oviposit in hosts that have been previously parasitized by *N. vitripennis*. Both studies suggest frequent multiparasitization of these two species in areas of sympatry, which will reduce the isolating effect of WHM. Leonard and Boake (2006) reported a correlation between aggression and site fidelity in males and the frequency of WHM. They proposed that WHM and decreased male aggression evolved in *N. giraulti* in response to the spread of *N. vitripennis* in North America. As *N. giraulti* males do not defend their exit holes as fiercely as *N. vitripennis*, females may disperse directly upon emergence, preventing the opportunity for *N. giraulti* males to mate them. This would give an advantage to males that mate females before emerging, by keeping them inside the host longer and creating the exit holes after mating. This explanation is however not at rhyme with the preference of *N. giraulti* for *N. vitripennis* pre-parasitized hosts. Instead, it should favour *N. giraulti* females that oviposit on hosts that are not already parasitized by *N. vitripennis*. More detailed observations of mating interactions between *N. vitripennis* and *N. giraulti* in the field are required to settle this issue.

Differences in mating behaviour are important for the build-up of reproductive isolation (Coyne and Orr 2004). In areas of sympatry, prezygotic isolation barriers help to prevent hybridization between species. The sympatric range of three *Nasonia* species in eastern North America (Darling and Werren 1990, Raychoudhury et al. 2010) provides a unique opportunity to study the evolution of these prezygotic isolation mechanisms. *N. vitripennis* females show strong mate discrimination against *N. giraulti* males, but *N. giraulti* females readily accept *N. giraulti* males (Giesbers et al. 2013; Buellesbach et al. 2014), which allows for hybridization. In contrast to previous studies that suggested that within-host-mating might have evolved in *N. giraulti* to prevent interspecific matings, we believe that

other factors may be more important to prevent interspecific matings in nature. Likely candidates are differences in cuticular hydrocarbon profiles (Niehuis et al 2011, Buellesbach et al 2013) and mating pheromones (Steiner et al 2006, Ruther et al 2007, 2014, Niehuis et al 2013).

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Table S5.1. Overview of the tested field-collected North-American *Nasonia* lines. Location indicates the town from which the wasps were collected (see text for details). Collection method indicates if an iso-female line was set up from a single female caught in the field (F) or from a single female that emerged from a host collected in the field (H). The within-host-mating (WHM) rate is the average for all tested females line.

Species	Isofemale line	Location	Nestbox	Collection method	# females tested	WHM index
N.vitripennis	DAV 1228_A	Davidson, NC	1228	H	84	0.231
	DAV 1228_C	Davidson, NC	1228	H	77	0.026
	DAV 1228_D	Davidson, NC	1228	H	55	0.000
	DAV 125_D	Davidson, NC	125	H	23	0.000
	DAV 125_F	Davidson, NC	125	H	19	0.111
	DAV 142_F	Davidson, NC	142	H	19	0.000
	DAV 142_G	Davidson, NC	142	H	28	0.000
	DAV 735_C	Davidson, NC	735	H	38	0.000
	DAV 735_G	Davidson, NC	735	H	22	0.333
	DAV 768_B	Davidson, NC	768	H	20	0.000
	DAV 768_D	Davidson, NC	768	H	37	0.000
	ITH 10_B	Ithaca, NY	10	H	19	0.000
	ITH 103_C	Ithaca, NY	103	H	28	0.000
	ITH 116_Z	Ithaca, NY	116	H	17	0.000
	ITH 124_A	Ithaca, NY	124	H	75	0.000
	ITH 124_B	Ithaca, NY	124	H	27	0.000
	ITH 130_I	Ithaca, NY	130	H	30	0.033
	ITH 130A I/F	Ithaca, NY	130	F	10	0.000
	ITH 130B I/F	Ithaca, NY	130	F	48	0.060
	ITH 130B I/F bait	Ithaca, NY	130	F	29	0.067
	ITH 138_A	Ithaca, NY	138	H	20	0.100
	ITH 139A I/F bait	Ithaca, NY	139	F	50	0.000
	ITH 140A I/F bait	Ithaca, NY	140	F	20	0.250
	ITH 145_C	Ithaca, NY	145	H	8	0.000
	ITH 148_E	Ithaca, NY	148	H	27	0.067
	ITH 157_C	Ithaca, NY	157	H	10	0.000
	ITH 157B I/F	Ithaca, NY	157	F	30	0.000
	ITH 157D I/F bait	Ithaca, NY	157	F	30	0.000
	ITH 16_J	Ithaca, NY	16	H	49	0.000
	ITH 22_A	Ithaca, NY	22	H	26	0.000

	ITH 69_E	Ithaca, NY	69	H	49	0.062
	ITH 82A I/F bait	Ithaca, NY	82	F	47	0.064
	ITH 85_F	Ithaca, NY	85	H	29	0.185
	ITH 93_D	Ithaca, NY	93	H	67	0.084
	ITH 95_M	Ithaca, NY	95	H	24	0.133
	ITH 98A I/F bait	Ithaca, NY	98	F	19	0.000
	ITH B130_A	Ithaca, NY	130	B	20	0.000
	ITH B134_C	Ithaca, NY	134	B	23	0.033
	ITH B153_A	Ithaca, NY	153	B	37	0.059
	ITH B3_C	Ithaca, NY	3	B	17	0.056
	ITH B91_A	Ithaca, NY	91	B	35	0.400
	LAN 62_B	Lancaster, PA	62	H	20	0.000
	LAN 62_D	Lancaster, PA	62	H	25	0.300
	LAN 62_E	Lancaster, PA	62	H	10	0.000
	LAN 62_G	Lancaster, PA	62	H	39	0.236
	LAN 62_I	Lancaster, PA	62	H	9	0.000
	LAN 62_L	Lancaster, PA	62	H	28	0.117
	LAN 62_M	Lancaster, PA	62	H	29	0.000
	LAN 62_Q	Lancaster, PA	62	H	9	0.000
	LAN 62_V	Lancaster, PA	62	H	11	0.083
	LAN 62_Z	Lancaster, PA	62	H	23	0.000
	QUBS 11_B	Kingston, ON, Canada	11	H	19	0.500
	QUBS 11_D	Kingston, ON, Canada	11	H	97	0.051
	QUBS 11_F	Kingston, ON, Canada	11	H	36	0.125
	QUBS 11_I	Kingston, ON, Canada	11	H	39	0.025
	QUBS 16_B	Kingston, ON, Canada	16	H	3	0.333
	QUBS 16_G	Kingston, ON, Canada	16	H	10	0.200
	QUBS 16_H	Kingston, ON, Canada	16	H	6	0.500
*N. girault*i	DAV 1113_C	Davidson, NC	1113	H	10	1.000
	DAV 1118_A I/F	Davidson, NC	1118	F	40	0.925
	DAV 1118_B I/F	Davidson, NC	1118	F	43	1.000
	DAV 1119_A	Davidson, NC	1119	H	18	0.950
	DAV 1119_C	Davidson, NC	1119	H	16	1.000
	DAV 121_B	Davidson, NC	121	H	10	1.000
	DAV 121_D	Davidson, NC	121	H	10	1.000

DAV 1228_H	Davidson, NC	1228	H	30	1.000
DAV 137_B	Davidson, NC	137	H	18	0.944
DAV 137_G	Davidson, NC	137	H	47	0.923
DAV 142_C	Davidson, NC	142	H	9	1.000
DAV 143_D	Davidson, NC	143	H	10	1.000
DAV 143_G	Davidson, NC	143	H	9	1.000
DAV 145_A I/F	Davidson, NC	145	F	49	0.898
DAV 174_H	Davidson, NC	174	H	42	1.000
DAV 174_I	Davidson, NC	174	H	59	0.981
DAV 182_B	Davidson, NC	182	H	19	1.000
DAV 182_E	Davidson, NC	182	H	8	1.000
DAV 309_A	Davidson, NC	309	H	27	0.967
DAV 309_C	Davidson, NC	309	H	8	1.000
DAV 309_D	Davidson, NC	309	H	10	0.900
DAV 309_F	Davidson, NC	309	H	38	0.944
DAV 320_H	Davidson, NC	320	H	45	0.980
DAV 320_I	Davidson, NC	320	H	61	0.907
DAV 415_C	Davidson, NC	415	H	10	1.000
DAV 423_B	Davidson, NC	423	H	10	1.000
DAV 423_C	Davidson, NC	423	H	10	1.000
DAV 423_E	Davidson, NC	423	H	10	0.900
DAV 423_F	Davidson, NC	423	H	18	1.000
DAV 52_B	Davidson, NC	52	H	20	1.000
DAV 52_C	Davidson, NC	52	H	9	0.889
DAV 539_F	Davidson, NC	539	H	10	1.000
DAV 568_A	Davidson, NC	568	H	10	0.300
DAV 568_B	Davidson, NC	568	H	11	0.222
DAV 65_D	Davidson, NC	65	H	69	0.986
DAV 652_H	Davidson, NC	652	H	18	0.788
DAV 68_H	Davidson, NC	68	H	10	0.900
DAV 701_D	Davidson, NC	701	H	36	0.894
DAV 701_E	Davidson, NC	701	H	35	0.975
DAV 701_F	Davidson, NC	701	H	10	0.900
DAV 715_A	Davidson, NC	715	H	30	0.950
DAV 715_F	Davidson, NC	715	H	18	1.000

	DAV 722_B	Davidson, NC	722	H	10	1.000
	DAV 722_C	Davidson, NC	722	H	7	0.571
	DAV 735_C	Davidson, NC	735	H	33	1.000
	DAV 77_B	Davidson, NC	77	H	30	0.967
	DAV 77_F	Davidson, NC	77	H	13	0.613
	DAV 78_D	Davidson, NC	78	H	10	0.900
	DAV 78_E	Davidson, NC	78	H	17	0.664
	DAV 78_G	Davidson, NC	78	H	20	1.000
	DAV 780_F	Davidson, NC	780	H	28	0.775
	DAV 780_H	Davidson, NC	780	H	9	0.889
	DAV 783_F	Davidson, NC	783	H	9	0.111
	DAV 783_I	Davidson, NC	783	H	19	0.789
	DAV 790_A	Davidson, NC	790	H	10	0.800
	DAV 792_A	Davidson, NC	792	H	10	1.000
	DAV 792_C	Davidson, NC	792	H	8	1.000
	DAV 792_F	Davidson, NC	792	H	8	1.000
	DAV 811_B	Davidson, NC	811	H	18	1.000
	DAV 811_C	Davidson, NC	811	H	30	1.000
	DAV 820_C	Davidson, NC	820	H	50	0.980
	DAV 823_D	Davidson, NC	823	H	10	0.900
	DAV 867_B	Davidson, NC	867	H	10	1.000
	ITH 103.17_E	Ithaca, NY	103	H	28	0.663
	WAY 130_G	Waynesboro, VA	130	H	65	0.857
	WAY 130_L	Waynesboro, VA	130	H	27	0.925
	WAY 130_M	Waynesboro, VA	130	H	39	0.917
	WAY 130_N	Waynesboro, VA	130	H	39	1.000
	WAY 139_A	Waynesboro, VA	139	H	10	0.900
	WAY 139_F	Waynesboro, VA	139	H	20	0.900
	WAY 139_H	Waynesboro, VA	139	H	20	0.900
	WAY 139_I	Waynesboro, VA	139	H	28	0.863
	WAY 139_X	Waynesboro, VA	139	H	38	0.922
	WAY 139_Z	Waynesboro, VA	139	H	10	1.000
	WAY 97_E	Waynesboro, VA	97	H	9	0.889
	WAY 97_K	Waynesboro, VA	97	H	9	1.000
N. longicornis	ITH 2.3_D	Ithaca, NY	2	H	50	0.260

ITH 2.3_E	Ithaca, NY	2	H	61	0.559
ITH 2.6_A	Ithaca, NY	2	H	48	0.393
ITH 2.6_C	Ithaca, NY	2	H	43	0.360

CHAPTER 5